REMARKS

The Office Action of October 20, 2004 presents the examination of claims 34-52. The present paper amends claims 34, 36, 37, 46 and 47 and adds new claims 53-59 for examination.

The Amendments to the Claims

Amended claim 34 and newly added claims 53-59 are slightly broader in scope than the previously pending claims 34, etc. The scope of the promoter driving the B- or T-cell receptor-encoding polynucleotide is recited as "cardiac tissue-specific" in these claims. Support for this amendment is provided in the specification at, for example, page 6, lines 5-6, which indicate that a receptor to be located on the surface of differentiating cells is to be expressed from an "organ-specific or tissue-specific promoter". The MLC-2 promoter is shown as a cardiac tissue-specific promoter in the table at the top of page 7.

Claims 34, etc. are amended to recite that the enhancer combined with the tissue-specific promoter is "constitutive", rather than one that is "operative in a mammalian embryonic stem cell, primordial cell or bone marrow stromal cell". This clarifies the nature of the enhancer and makes its scope commensurate with the intended function of the promoter-enhancer as is explained in detail below. Claims 34, etc. are also amended to recite that the promoter that drives expression of the selection marker used at the

step of initial transformation of the cells is operable in ES, primordial or stromal cells, rather than "constitutively" operable in such cells. Again, this conforms the language of the claims to the required function of the promoter that is plainly set forth in the specification.

Finally, claim 34 is amended to clarify the arrangement of the LoxP sequences in relation to the promoter-selectable marker element of some embodiments of the construct. See, for example, Figure 1 and page 12, last line to page 13, first line, and the last paragraph of page 14.

Dependency of claims is changed to avoid increasing fees for additional claims.

Rejection under 35 U.S.C. § 112, first paragraph

Claims 34-36 and 50-52 stand rejected under 35 U.S.C. § 112, first paragraph, for alleged lack of adequate written description of certain of their claim terms in the specification. This rejection is respectfully traversed. Reconsideration and withdrawal thereof are requested.

The Examiner asserts that there is lack of written description of the claim elements of a genus of enhancers operative in a mammalian embryonic stem cell, bone marrow stromal cells and primordial cells. The Examiner considers that description of the CMV enhancer as an example of an enhancer, and of a PGK promoter as

an example of a promoter, are not sufficient to support this generic recitation in the claims.

As a threshold matter, as explained above, the present claims have been amended to delete recitation of a "constitutive enhancer operative in mammalian ES cells…". Applicant believes the present claim language is well-supported by the specification.

Should the Examiner require further explanation, the Examiner is reminded that the legal test for sufficiency of the written description is merely that the specification must show to one of ordinary skill in the art that the inventor, at the time of filing of the application, was in possession of the subject matter claimed. *Vas-Cath Inc. v. Mahurkar*, 19 USPQ2d 1111 (Fed. Cir. 1991). It is not necessary that the exact words of the claims be present in the specification (see, *e.g. In re Gosteli*, 10 USPQ2d 1614 (Fed. Cir. 1989)); the showing is one from the specification as a whole, including the drawings. *Vas-Cath,* at p. 1118.

Furthermore, it is not necessary that the specification describe what is already known to one of ordinary skill in the art. *Webster Loom Co. v. Higgins*, 105 U.S. 580, 585-86 (1882), quoted in *Ralston Purina Company v. Far-Mar-Co, Inc.*, 222 USPQ 863 (DC Kan 1984). "(As the Court observed in Webster Loom, the applicant 'may begin at the point where his invention begins, and describe what he has made that is new, and what it replaces of the old. That which is common and well known

is as if it were written out in the patent and delineated in the drawings.' Id. at 586.")

Applicant's prior explanation to the Examiner in this regard is repeated below for convenient review by the reader and Applicant again urges that this explanation establishes that they had in their possession as of the filing date of the present application the invention as now claimed.

> The Examiner takes a position that, as to the promoter to be used at the step of selecting transformed cells, the generic nature of the invention was not adequately described in the original application. Similarly, the Examiner asserts that the specification fails to provide any generic description of a "constitutive" enhancer.
>
> Applicant disagrees. Applicant has specifically indicated in their previous Amendment where support for each limitation in the new claims is provided by the specification. This specifically included mention of support for a constitutive enhancer as a genus. Applicant again notes the generic concept of an enhancer is mentioned at, *e.g.*, page 10, line 5 of the specification and the CMV enhancer is mentioned as a specific example of such at, *e.g.*, page 13, line 3. Applicant furthermore asserts that the artisan of ordinary skill would recognize that the CMV enhancer is a strong, constitutive enhancer. This is evidenced by the attached Exhibit 1, a page from a catalog of a molecular biology supply company, which describes a vector including a CMV promoter/enhancer as one that "allows strong constitutive expression in many cell types."
> The specification also well-describes the aspect of the invention that the promoter operative in mammalian ES cells, primordial cells or bone stromal cells is used to drive expression of the selectable marker gene used for selecting initially transformed cells. Thus, Applicant also considers that the skilled artisan would recognize the generic nature of the promoter used at the step from the example of the PGK promoter and the text at, *e.g.* the last paragraph on page 10 to the first paragraph on page 11 (which describes the initial selection step) and the

> text at page 20, lines 19-21 (describing use of the PGK promoter for use in driving the gene for initial selection of transformed ES, primoridial or bone marrow stromal cells). The Examiner should also consider Figure 1, which shows that the first step in the transformation process is one in which selection of transformed human or murine embryonal stem cells (*i.e.* ES cells) is performed by selection using an antibiotic, resistance to which is expressed by a gene under the control of a PGK promoter. ("CMV-neo" is the name of the vector as a whole.) Finally, the Lallemand reference cited by the Examiner as prior art describes the PGK promoter as one that is active in the primordial germ cell.

To this earlier argument, Applicant would add that at page 10, in the last paragraph, selection of initially transformed cells by a selectable marker is described. At page 11, lines 1-2, there is the phrase, "Preferably, the resistance gene and its assigned promoter...". This plainly shows that a resistance gene to be utilized for selecting initially transformed cells is to be driven by an independent promoter. Figure 1 shows that the cells initially transformed are "murine or embryonal stem cells or bone marrow cells". Furthermore, at page 13, lines 20-22 are mentioned "primordial cells" and "bone marrow stroma cells" as cells to be transformed with the construct of the invention. Figure 1 shows that a neomycin resistance gene is linked to a PGK promoter, which is described by Applicant as one example of a promoter active in ES cells, primordial cells or bone marrow stromal cells. Applicant submits that the above disclosure well establishes that Applicant had conceived of their invention as one in which an event of

transformation of a ES or primordial or bone marrow stromal cells was to be selected for by use of a selectable marker gene (*e.g.* an antibiotic resistance) driven by a promoter operative in these cells types.

The specification provides the PGK promoter as an example of one promoter that is operative in ES cells, primordial cells or bone marrow stromal cells. At the time of filing of the application, additional such promoters were well-known to one of ordinary skill in the art. Applicant provides the attached Exhibits 3 and 4, abstracts of Barnea et al. and Fukushima et al., published prior to the filing date of the instant application, showing that the skilled artisan would also know that the promoters of the UTF1 gene and of the Oct 3/4 gene are operative in ES cells.

Applicant submits that the above explanation of the text of the specification and evidence of the knowledge of one of ordinary skill in the art is sufficient to show that the terms describing the promoter operatively linked to the selection marker in the claims are adequately supported by the specification.

The present claims also recite a cardiac tissue-specific promoter, or "promoter-enhancer" that is operatively linked to a polynucleotide encoding a portion of a B- or T-cell receptor to drive its expression. The present claims recite that the enhancer linked to the tissue-specific promoter is described as "constitutive".

In this regard, the Examiner should consider in addition to Applicant's previous explanation that the specification provides description of the invention in broad generic terms. The full breadth of the invention set forth is of a construct that is useful for selecting from cultured ES, primordial or stromal cells those cells which have differentiated to a state that a promoter having the property of specifically expressing a gene in a desired tissue is recognized and activated. That is, a gene providing for separation of desired cells from undesired cells (*i.e.* the "receptor" gene) is expressed under the control of a tissue-specific promoter when the ES cells, primordial cells or stromal cells that are transformed with the construct of the invention have differentiated into a form that activates the tissue-specific promoter. Then these desired cells can be separated from the undesired cells by a separation technique recognizing the "receptor" gene.

The specification describes that the particular tissue that can be derived from the technique is not limited, but can be chosen by an appropriate choice of tissue- or organ-specific promoter (*e.g.* at page 6, lines 14 ff.). The specification describes that the functions of the promoter driving the "receptor"-encoding polynucleotide that are important to utility are that it be tissue- or organ-specific and that it be effective in expressing the

"receptor"-encoding polynucleotide "after induction of cell differentiation" (see page 6, line 10).

The above description urges one of ordinary skill in the art to consider the invention to be broad in scope and thus to consider the various known promoters and enhancers as alternatives to the specific examples set forth as the working example in the specification. Applicant further notes the admission of the Examiner at the bottom of page 11 of the Office Action that "numerous" cardiac-specific promoters were known at the time the application was filed. As further support for this proposition, Applicant provides Exhibits 5-8, showing that the promoters of the Flk-1 gene (VEGF receptor-2), eNOS gene and cardiac α-actin gene were known to the skilled artisan at the time of filing of the present application as cardiac tissue-specific promoters.

The specification further describes that promoters used in the expression cassette may be optionally combined with other regulatory elements, such as an enhancer, *e.g.* at page 10, line 5.

One of ordinary skill in the art would thus understand that it may be necessary to link some tissue-specific promoters to an enhancer to assure that it is strong enough to perform the function of expressing the "receptor"-encoding polynucleotide "after induction of cell differentiation". The artisan of ordinary skill would also understand, given the example of the CMV enhancer described in the specification, that a constitutive enhancer would

be useful in such a combination. Applicant has made evidence of record, that a CMV enhancer was known at the time the application was filed to be a constitutive promoter (Exhibit 1), and furthermore that a constitutive enhancer could be combined with a tissue-specific promoter to increase the strength of expression of a gene without sacrificing tissue-specificity of expression (Exhibit 2).

Applicant submits that the specification plainly shows to one of ordinary skill in the art that, at the time the application was filed, they were in possession of the invention as now claimed. Therefore, the rejection of claims 34-36 and 50-52 under 35 U.S.C. § 112, first paragraph, for alleged lack of written description in the specification, should be withdrawn.

Rejection Over Prior Art

Claims 34(A, B), 35, 38, 39, 40, 42, 46 and 50-52 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Klug, Gaines, Griscelli, and Franz, in view of Mack. The rejection is made "for reasons of record", claims 51-52 being included in the rejection on a theory of inherency.

This rejection is respectfully traversed. Reconsideration and withdrawal thereof are requested.

Applicant first again submits that the Examiner fails to properly establish *prima facie* obviousness of the claimed

invention. In particular, Applicant again submits that the arrangement of the angiogenesis factor-encoding polynucleotide for expression from an IRES sequence in the part of the cassette also encoding the tissue-specific cell type purification marker is not *prima facie* obvious.

The Examiner asserts that the skilled artisan reading Gaines would be motivated to utilize an IRES for simultaneous expression of VEGF, a gene disclosed by Mack as having therapeutic value in treatment of ischemic disease, simultaneously with another heterologous gene. However, the Examiner fails to establish, by reasoning or by evidence, that one of ordinary skill in the art would find it desirable to simultaneously express a therapeutic gene encoding an angiogenesis factor simultaneously with a marker used for selection of differentiating cells. Applicant further notes that Gaines, cited by the Examiner for the disclosure that an IRES should be used for expression of a therapeutic gene, in fact urges an arrangement in which the marker for separation of desired cells from undesired cells is driven by the IRES, while the second heterologous gene is expressed directly from a constitutive promoter (see Figure 1). The Examiner should note that the combination of Mack with Gaines therefore urges that a heterologous therapeutic gene and a selection marker should be driven together from a constitutive promoter, not from a promoter activated by cell differentiation. Accordingly, there is no motivation provided by

the cited references, or by the state of the art, to arrange the various elements of the construct in the manner presently claimed.

Also, the Examiner has not explained why the artisan of ordinary skill is motivated by the cited references, or by the state of the art, to specifically combine a polynucleotide encoding a receptor expressed on the surface of a B- or T-cell with a cardiac tissue-specific promoter. This is a second aspect of the claimed invention not motivated by the prior art of record.

Thus, the instantly claimed invention is not *prima facie* obvious, and the instant rejection should be withdrawn.

Applicant furthermore submits that the present invention provides results unexpected by one of ordinary skill in the art at the time the invention was made.

Applicant has previously pointed out the electrophysiology of the selected cells and the high purity of them obtained. The Examiner takes a position that, because Klug et al. use the same promoter as in the instant invention, they select the same cells and so the same physiology would be expected. Applicant notes that Klug utilize antibiotic selection, not cell sorting utilizing a specific receptor, to obtain their cells. Klug et al. do observe that a small percentage of the transformed cells, prior to selection, exhibit contractile activity, but dissected contractile regions are shown to be composed of but a small percentage of cardiomyocytes (Table 1). Klug also show by immunoreactivity that

selected cells express cardiomyocyte marker proteins and, also by immunoreactivity, that engrafted selected cells remain viable in the heart tissue. However, there is no evidence in Klug that the contractile activity is due to cardiomyocytes in the culture, nor is there any evidence that the engrafted cells in the heart exhibit any contractile or electrical activity. Thus, there is no evidence that that the G418 selected cells in fact exhibit the electrophysiologic properties of the cells obtained using the instant invention.

The Examiner is reminded that a theory of inherency requires that the result relied upon be a necessary, not merely possible or even probable, outcome of the structure or method used in the reference. *In re Betz*, 77 USPQ 162 (CCPA 1948). The process utilized by Klug to obtain their cells is different from that used by the Applicant. Therefore, while the Applicant can agree that it is possible that Klug's cells exhibit the electrophysiology of the cells of the instant invention, this does not rise to the standard of necessity that is required for the Examiner to rely upon it to assert Klug's cells inherently exhibit the same electrophysiology as Applicant's cells and therefore that the result asserted by Applicant as unexpected is in fact already obtained in the prior art.

Second, the Applicant provides attached hereto as Exhibit 9 a letter (and an English translation) from a reviewer of a proposal

made by Dr. Franz to import human ES cells into Germany for further experimentation. The Examiner should take note that the reviewer indicates that some "uncertainties" remain about the feasibility of the method for producing and enriching CD4-positive cardiomyocytes from ES cells. To reply to these uncertainties, Dr. Franz is requested to provide further showings that, "these separated, differentiated CD4-positive mouse cells are showing the expected in vitro differentiated characteristics of cardiomyocytes (*e.g.*, action potentials, expression of cardiomyocyte specific proteins)." (Emphasis added.)

A reviewer of an experimental protocol for feasibility is one of ordinary skill in the art. The letter of Exhibit 9 thus constitutes an opinion of one of ordinary skill in the art that, as of December 16, 2002, almost two years after the priority date of the instant application, it was not expected that cardiac myocytes cells prepared by *in vitro* differentiation of ES cells would exhibit proper electrophysiology. However, this result is unambiguously obtained by the use of the construct of the present invention, as shown in Figure 2 of the specification. Accordingly, the present invention should be considered unobvious over the cited references and the instant rejection should be withdrawn.

Claims 34(C-E), 36, 37, 41 and 43-45 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Klug, Gaines, Griscelli,

Franz and Mack as applied previously, in further view of Graham, Gainer and Lallemand. This rejection is respectfully traversed. Reconsideration and withdrawal thereof are requested.

The Graham, Gainer and Lallemand references are cited in addition to the others as describing further elements of the invention. Graham is cited for disclosure of the LoxP system and its use in excision of selectable marker genes. Lallemand is cited for disclosure of a PGK promoter and its use to drive expression of a selectable marker gene. Gainer is cited for disclosure of a CTLA-4 gene and its use in immunosuppression.

None of these references provide any disclosure that remedies the deficiencies of the combination of Klug, Gaines, Griscelli, Franz and Mack in establishing *prima facie* obviousness of the invention as explained above. Thus, the instant rejection should be withdrawn for the same reasons as set forth above in addressing the rejection based only on the first group of references.

Reconsideration of Rejoiner

The Examiner maintains claims 47-49 as withdrawn from examination. Applicant again urges reconsideration of withdrawal of these claims from examination.

The Examiner's main reason for maintaining that claims 47-49 should not be examined is that,

> [T]he elected invention is drawn to transfecting pluripotent stem cells with a cassette having promoters that only function[s] in either said stem cells or a cardiomyocyte. Thus, claiming any cell transfected with such expression cassette would lead to a different invention drawn to cells with different utility and require different search and technical consideration.

This reasoning ignores two limitations in claims 47-49. The first ignored limitation is that the claimed cells comprise the cassette having all of the elements that are recited in one of the claims presently under consideration. The cassette is one that has the activity of differentiating ES cells into cardiomyocytes and/or selecting cells differentiating into cardiomyocytes, from cultured ES cells. The second ignored limitation is recited in claim 47, which expressly states that the claimed cell is one "exhibiting the electrophysiological properties similar to those of a ventricular cardiomyocyte." Therefore, the invention of claim 47 is not susceptible to different utility depending upon the particular cell that is transformed. The claimed cell is one that has utility as a ventricular cardiomyocyte.

For the above reasons, Applicant again urges the Examiner to reconsider rejoinder of claims 47-49 to the instant application for examination.

Conclusion

The present application well-describes and claims patentable subject matter. The favorable action of allowance of the pending

claims and passage of the application to issue is respectfully requested.

Pursuant to the provisions of 37 C.F.R. §§ 1.17 and 1.136(a), Applicant respectfully petitions for a three (3) month extension of time for filing a response in connection with the present application. The required fee of $510.00 is attached hereto.

Should there be any outstanding matters that need to be resolved in the present application, the Examiner is respectfully requested to contact Mark J. Nuell (Reg. No. 36,623) at the telephone number of the undersigned below, to conduct an interview in an effort to expedite prosecution in connection with the present application.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. §§ 1.16 or 1.17; particularly, extension of time fees.

Respectfully submitted,

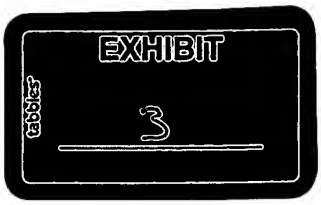
BIRCH, STEWART, KOLASCH & BIRCH, LLP

By _____
Mark J. Nuell, #36,623

P.O. Box 747
Falls Church, VA 22040-0747
(703) 205-8000

DRN/mua
0690-0115P

Attachment(s): Exhibits 3-9



J Biol Chem. 2000 Mar 3;275(9):6608-19. Related Articles, Links

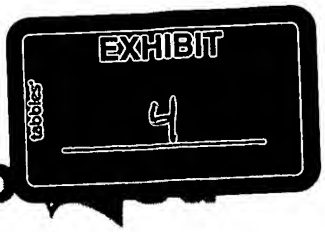
Synergy of SF1 and RAR in activation of Oct-3/4 promoter.

Barnea E, Bergman Y.

The Hubert H. Humphrey Center for Experimental Medicine and Cancer Research, The Hebrew University, Hadassah Medical School, Jerusalem 91120, Israel.

The Oct-3/4 transcription factor is expressed in the earliest stages of embryogenesis, and is thus likely to play an important role in regulation of initial decisions in development. For the first time, we have shown that SF1 and Oct-3/4 are co-expressed in embryonal carcinoma (EC) P19 cells, and their expression is down-regulated with very similar kinetics following retinoic acid (RA) induced differentiation of these cells, suggesting a functional relationship between the two. Previously, we have shown that the Oct-3/4 promoter harbors an RA-responsive element, RAREoct, which functions in EC cells as a binding site for positive regulators of transcription, such as RAR and RXR. In this study we have identified in the Oct-3/4 promoter two novel SF1-binding sites: SF1(a) and SF1(b). The proximal site, SF1(a), is located within the RAREoct, and the distal site, SF1(b), is located between nucleotide -193 and -209 of the Oct-3/4 promoter. Both sites contribute to activation of Oct-3/4 promoter in EC cells, with SF1(a) playing a more crucial role. SF1, and its isoforms ELP2 and ELP3 bind to both SF1 sites and activate the Oct-3/4 promoter. This activation depends on the presence of SF1 DNA-binding domain. Thus, Oct-3/4 is the first EC-specific gene reported that is regulated by SF1. Interestingly, SF1 and RAR form a novel complex on the RAREoct sequence that synergistically activate the Oct-3/4 promoter. Both RARE and SF1 cis regulatory elements, as well as the SF1 DNA-binding domain, are needed for this synergism. SF1 and Oct-3/4 transcription factors play a role in the same developmental regulatory cascade.

PMID: 10692469 [PubMed - indexed for MEDLINE]



1: Mol Cell Biol. 1999 Aug;19(8):5453-65. Related Articles, Links

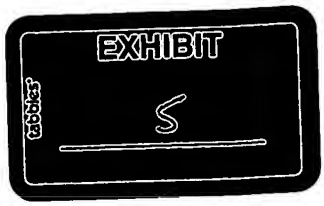
The gene for the embryonic stem cell coactivator UTF1 carries a regulatory element which selectively interacts with a complex composed of Oct-3/4 and Sox-2.

Nishimoto M, Fukushima A, Okuda A, Muramatsu M.

Department of Biochemistry, Saitama Medical School, Iruma-gun, Saitama 350-0495, Japan.

UTF1 is a transcriptional coactivator which has recently been isolated and found to be expressed mainly in pluripotent embryonic stem (ES) cells (A. Okuda, A. Fukushima, M. Nishimoto, et al., EMBO J. 17:2019-2032, 1998). To gain insight into the regulatory network of gene expression in ES cells, we have characterized the regulatory elements governing UTF1 gene expression. The results indicate that the UTF1 gene is one of the target genes of an embryonic octamer binding transcription factor, Oct-3/4. UTF1 expression is, like the FGF-4 gene, regulated by the synergistic action of Oct-3/4 and another embryonic factor, Sox-2, implying that the requirement for Sox-2 by Oct-3/4 is not limited to the FGF-4 enhancer but is rather a general mechanism of activation for Oct-3/4. Our biochemical analyses, however, also reveal one distinct difference between these two regulatory elements: unlike the FGF-4 enhancer, the UTF1 regulatory element can, by its one-base difference from the canonical octamer-binding sequence, selectively recruit the complex comprising Oct-3/4 and Sox-2 and preclude the binding of the transcriptionally inactive complex containing Oct-1 or Oct-6. Furthermore, our analyses reveal that these properties are dictated by the unique ability of the Oct-3/4 POU-homeodomain that recognizes a variant of the Octamer motif in the UTF1 regulatory element.

PMID: 10409735 [PubMed - indexed for MEDLINE]



1: Blood. 1999 Jun 15;93(12):4284-92. Related Articles, Links

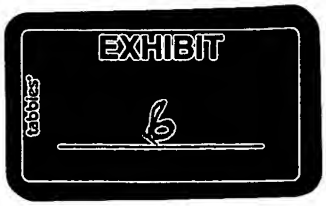
Identification of vascular endothelial growth factor (VEGF) receptor-2 (Flk-1) promoter/enhancer sequences sufficient for angioblast and endothelial cell-specific transcription in transgenic mice.

Kappel A, Ronicke V, Damert A, Flamme I, Risau W, Breier G.

Max-Planck-Institute for Physiological and Clinical Research, Bad Nauheim, Germany; the Zentrum fur molekulare Medizin, Koln, Germany.

The vascular endothelial growth factor (VEGF) receptor-2 (Flk-1) is the first endothelial receptor tyrosine kinase to be expressed in angioblast precursors, and its function is essential for the differentiation of endothelial cells and hematopoietic precursors. We have identified cis-acting regulatory elements of the murine Flk-1 gene that mediate endothelium-specific expression of a LacZ reporter gene in transgenic mice. Sequences within the 5'-flanking region of the Flk-1 gene, in combination with sequences located in the first intron, specifically targeted transgene expression to angioblasts and endothelial cells of transgenic mice. The intronic regulatory sequences functioned as an autonomous endothelium-specific enhancer. Sequences of the 5'-flanking region contributed to a strong, uniform, and reproducible transgene expression and were stimulated by the transcription factor HIF-2alpha. The Flk-1 gene regulatory elements described in this study should allow the elucidation of the molecular mechanisms involved in endothelial cell differentiation and angiogenesis.

PMID: 10361126 [PubMed - indexed for MEDLINE]



Guillot PV, Liu L, Kuivenhoven JA, Guan J, Rosenberg RD, Aird WC. Related Articles, Links
Targeting of human eNOS promoter to the Hprt locus of mice leads to tissue-restricted transgene expression.
Physiol Genomics. 2000 Mar 13;2(2):77-83.
PMID: 11015585 [PubMed - indexed for MEDLINE]

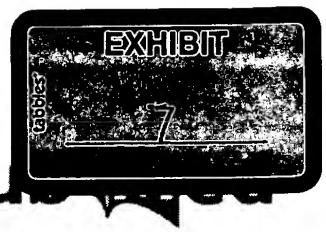
Targeting of human eNOS promoter to the Hprt locus of mice leads to tissue-restricted transgene expression.

Guillot PV, Liu L, Kuivenhoven JA, Guan J, Rosenberg RD, Aird WC.

Beth Israel Deaconess Medical Center, Department of Molecular Medicine, Boston 02215, USA.

Phenotypic heterogeneity of the endothelium arises from cell type-specific differences in gene expression. An understanding of the mechanisms that underlie differential gene expression would provide important insight into the molecular basis of vascular diversity. In standard transgenic assays, multiple copies of heterologous DNA cassettes are randomly integrated into the mouse genome, resulting in significant line-to-line variation in expression. To overcome these limitations, we have targeted a single copy of a transgene that contains 1,600 bp of the human endothelial nitric oxide synthase (eNOS) promoter coupled to the LacZ reporter gene to the X-linked hypoxanthine phosphoribosyltransferase (Hprt) locus of mice by homologous recombination. The transgene was inserted in either of the orientations relative to that of the Hprt gene. In mice derived from multiple embryonic stem (ES) cell clones, the expression pattern was limited to a subset of endothelial cells, cardiomyocytes, and vascular smooth muscle cells. These findings suggest that Hprt locus targeting is a feasible tool for studying endothelial cell-restricted gene regulation.

PMID: 11015585 [PubMed - indexed for MEDLINE]



Limits: **Publication Date from 1990/01/01 to 2000/03/10**

1: Cells Tissues Organs. 1999;165(3-4):153-64. Related Articles, Links

Full Text

Establishment of ionic channels and signalling cascades in the embryonic stem cell-derived primitive endoderm and cardiovascular system.

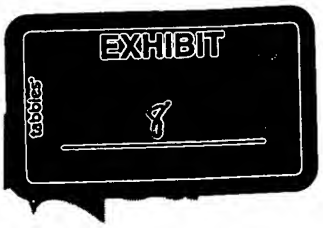
Hescheler J, Fleischmann BK, Wartenberg M, Bloch W, Kolossov E, Ji G, Addicks K, Sauer H.

Institute of Neurophysiology, University of Cologne, Germany.

The first organ system to be established in early embryogenesis is the cardiovascular system which develops upon interaction with hypoblastic cells of the primitive endoderm. Here we focus on recent work on embryoid bodies derived from pluripotent embryonic stem (ES) cells. Ca(2+) oscillations and Ca(2+) signalling pathways during the differentiation of primitive endodermal cell layers are reported. Furthermore, the development-dependent expression of ion channels and the buildup of signalling cascades involved in the modulation of voltage-dependent L-type Ca(2+) channels during early cardiomyogenesis and the formation of functional vascular structures in the process of vasculogenesis and angiogenesis are reviewed. We also report on the use of green fluorescent protein reporter gene expression under the control of cardiac-specific promoters, e.g. the human cardiac alpha-actin promoter, which enables the identification and in vivo characterization of cardiomyocytes at very early stages of cardiomyogenesis.

Publication Types:
- Review
- Review, Tutorial

PMID: 10592387 [PubMed - indexed for MEDLINE]



1: J Cell Biol. 1998 Dec 28;143(7):2045-56. Related Articles, Links

FREE full text article at www.jcb.org

Functional characteristics of ES cell-derived cardiac precursor cells identified by tissue-specific expression of the green fluorescent protein.

Kolossov E, Fleischmann BK, Liu Q, Bloch W, Viatchenko-Karpinski S, Manzke O, Ji GJ, Bohlen H, Addicks K, Hescheler J.

Institute of Neurophysiology, University of Cologne, D-50931 Cologne, Germany.

In contrast to terminally differentiated cardiomyocytes, relatively little is known about the characteristics of mammalian cardiac cells before the initiation of spontaneous contractions (precursor cells). Functional studies on these cells have so far been impossible because murine embryos of the corresponding stage are very small, and cardiac precursor cells cannot be identified because of the lack of cross striation and spontaneous contractions. In the present study, we have used the murine embryonic stem (ES, D3 cell line) cell system for the in vitro differentiation of cardiomyocytes. To identify the cardiac precursor cells, we have generated stably transfected ES cells with a vector containing the gene of the green fluorescent protein (GFP) under control of the cardiac alpha-actin promoter. First, fluorescent areas in ES cell-derived cell aggregates (embryoid bodies [EBs]) were detected 2 d before the initiation of contractions. Since Ca2+ homeostasis plays a key role in cardiac function, we investigated how Ca2+ channels and Ca2+ release sites were built up in these GFP-labeled cardiac precursor cells and early stage cardiomyocytes. Patch clamp and Ca2+ imaging experiments proved the functional expression of the L-type Ca2+ current (ICa) starting from day 7 of EB development. On day 7, using 10 mM Ca2+ as charge carrier, ICa was expressed at very low densities 4 pA/pF. The biophysical and pharmacological properties of ICa proved similar to terminally differentiated cardiomyocytes. In cardiac precursor cells, ICa was found to be already under control of cAMP-dependent phosphorylation since intracellular infusion of the catalytic subunit of protein kinase A resulted in a 1.7-fold stimulation. The adenylyl cyclase

activator forskolin was without effect. IP3-sensitive intracellular Ca2+ stores and Ca2+-ATPases are present during all stages of differentiation in both GFP-positive and GFP-negative cells. Functional ryanodine-sensitive Ca2+ stores, detected by caffeine-induced Ca2+ release, appeared in most GFP-positive cells 1-2 d after ICa. Coexpression of both ICa and ryanodine-sensitive Ca2+ stores at day 10 of development coincided with the beginning of spontaneous contractions in most EBs. Thus, the functional expression of voltage-dependent L-type Ca2+ channel (VDCC) is a hallmark of early cardiomyogenesis, whereas IP3 receptors and sarcoplasmic Ca2+-ATPases are expressed before the initiation of cardiomyogenesis. Interestingly, the functional expression of ryanodine receptors/sensitive stores is delayed as compared with VDCC.

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TO Dr. Mark Nuell
- urgent -

EXHIBIT 9

DRN MRG

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Projekt Stammzellgesetz

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16.12.2002

Ihr Antrag auf Erteilung einer Genehmigung für Einfuhr und Verwendung humaner embryonaler Stammzellen

Ihr Zeichen

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Sehr geehrter Herr Dr. Franz,

es besteht nach Beratung in der Zentralen Ethik-Kommission für Stammzellforschung nach wie vor Unklarheit, ob die Verwirklichung der Methode zur Gewinnung und Anreicherung CD4-positiver Kardiomyozyten hinreichend in ES-Zellen der Maus validiert ist.
Sie werden daher nochmals gebeten, zu zeigen

1.) daß es Ihnen gelungen ist, murine ES-Zellen stabil mit dem MLC-2 (Rattenpromotor oder humaner Promotor)-CDH Expressionsvektor zu transfizieren,
2.) daß sich die transfizierten murinen ES-Zellen in vitro innerhalb der *embryoid bodies* (EB) differenzieren lassen,
3.) daß sich die murinen EB ohne Beeinflussung der Expression des CD4-Rezeptors auf der Zelloberfläche in Einzelzellen separieren und sich anschließend von CD4-negativen Zellen trennen lassen,
4.) daß die separierten, differenzierten CD4-positiven Mauszellen in vitro Charakteristika von Kardiomyozyten aufweisen (z.B. Aktionspotentiale, Expression Kardiomyozyten-spezifischer Proteine),

5.) daß die CD4-positiven murinen Kardiomyozyten im Tiermodell (Maus) nach Transplantation/Injektion in geschädigtes Myokard einwandern können, sich dort langfristig etablieren und funktionell aktiv sind. Wir bitten Sie, in diesem Zusammenhang auch um Darlegung Ihrer Kriterien der Funktionsüberprüfung.

Sie weisen in Ihrem Antrag (Seite 11 Pkt. 3.1) daraufhin, daß die Expression des humanen Oberflächenmoleküls CD4 nach Transplantation der Kardiomyozyten keine Immunreaktion auslösen kann. Welche Erkenntnisse bzw. vorliegenden Befunde stützen diese Annahme?

Für eine Übersendung Ihrer Darlegungen bis zum 17. Januar 2003 wären wir dankbar.

Mit freundlichen Grüßen
im Auftrag

i.V. Dr. Lerch

C. Lerch

Your proposal for allowance to import and use human embryonic stem cells

Dear Dr. Franz

We had discussions at the central ethic commission for stem cell science (Zentrale Ethik-Kommission für Stammzellforschung) and there are still some uncertainties in the context of the feasibility of your method for the production and enrichment of CD4 positive cardiomyocites in ES-cells of mice.

Therefore we ask you once again to show:

1) that it is possible to transfect the MLC-2 (rat or human promoter)-CD4 expression vector stably into murine ES cells,
2) that these transfected murine ES-cells will differentiate in vitro inside the embryoid bodies (EB),
3) that the murine EB can be separated into single cells without interfering the expression of the CD4-receptor at the cell surface, and that the CD4 positive population can be purified from the CD4 negative cells,
4) that these separated, differentiated CD4-positive mouse cells are showing the expected in vitro characteristics of cardiomyocytes (action potentials, expression of cardiomyocyte specific proteins),
5) and that the CD4-positive murine cardiomyocytes have shown in vivo (mouse) that they can be stably integrated into affected myocardium and have shown for a long time there normal activity. Please explain us in this context your criteria for testing these circumstance.

You have pointed out in your proposal (page 11, article 3.1) that the expression of the human surface molecule CD4 out of the transplanted cardiomyocytes will not lead to a response of the immune system. Which results or findings can corroborate this belief?

Please send us your explanation until 17th of January 2003